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Factors required for bone marrow stromal fibroblast colony formation *in vitro*

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Summary. Marrow stromal fibroblasts (MSFs) are essential for the formation of the haemopoietic microenvironment and bone; however, regulation of MSF proliferation is poorly understood. MSF colony formation was studied in primary mouse and human marrow cell cultures. After a brief exposure to serum, MSF colony formation occurred in the absence of both serum and non-adherent marrow cells, if medium conditioned by marrow cells was present (serum-free conditioned medium, SF-CM). In mouse and human cultures stimulated to proliferate by SF-CM, neutralizing antibodies against PDGF, TGF-β, bFGF and EGF specifically suppressed MSF colony formation. The degree of supression was species-dependent, with the most profound inhibition achieved in mouse cultures by anti-PDGF, anti-bFGF and anti-EGF, and in human cultures by anti-PDGF and anti-TGF-β. Serum-free medium not conditioned by marrow cells (SFM) did not support MSF colony formation. In mouse cultures in SFM, human recombinant bFGF and bovine natural bFGF were able to partially substitute for the stimulating effect of SF-CM. Other growth factors, including TGF-β_1, TGF-β_2, PDGF, EGF, IL-6, IGF-I and IGF-II, showed no activity when tested alone. In human cultures in SFM, none of the growth factors, alone or in combination, stimulated MSF colony formation. Mouse and human MSFs grown in SF-CM formed bone and a haemopoietic microenvironment when transplantated into immunodeficient mice *in vivo*, and therefore were functionally equivalent to MSFs generated in the presence of serum. These data indicate that stimulation of the initial proliferation of an MSF precursor cell is complex, and requires participation of at least four growth factors: PDGF, bFGF, TGF-β and EGF. In addition, mouse and human MSF precursor cells have different requirements for each of the growth factors.

Keywords. marrow stromal fibroblasts, colony formation, growth factors, neutralizing antibodies.

The term 'marrow stromal fibroblasts' (MSFs) designates a particular adherent cell type growing in bone marrow cultures *in vitro*. MSFs share a number of features in common with fibroblasts, and lack basic characteristics of endothelial cells and macrophages (Castro-Malaspina *et al*, 1980; Song & Quesenberry, 1984; Wang & Wolf, 1990; Zhang *et al*, 1995). Even after long-term cultivation and multiple passages *in vitro*, MSFs can form at least five types of connective tissue upon transplantation *in vivo*: haemopoiesis-supporting reticular stroma, adipose tissue, bone, cartilage, and fibrous tissue (Friedenstein *et al*, 1974; Owen, 1988; Beresford, 1989). Thus, MSF population contains stem cells capable of both prolonged proliferation and differentiation into several directions (Owen, 1988).

If a single cell suspension of bone marrow cells is plated *in vitro* at low plating density, discrete MSF colonies are formed. Each MSF colony is a clone produced by proliferation of a single precursor cell, a colony forming unit-fibroblast (CFU-F) (Friedenstein *et al*, 1978; Latsinik *et al*, 1986). CFU-Fs are cells residing in the bone marrow stromal compartment, and it is their *in vitro* progeny that are designated as MSFs. CFU-Fs are not related to the haemopoietic stem cells and represent a separate lineage(s) of a mesenchymal nature (Friedenstein *et al*, 1978; Golde *et al*, 1980). In steady-state conditions *in vivo*, CFU-Fs are for the most part in the G_0 stage of the cell cycle (Castro-Malaspina *et al*, 1980; Kanesko *et al*, 1982). In monolayer cultures, CFU-Fs enter into S phase between 24 and 60 h after explantation (Friedenstein *et al*, 1974), a parameter that may relate to the rate at which

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they begin to proliferate after bone marrow disturbances *in vivo*. An understanding of mechanisms controlling CFU-F proliferation may shed light on basic biological processes, such as formation of haemopoietic microenvironment and bone turnover, and have important clinical implications, including treatment of myelofibrosis, osteoporosis, and gene therapy.

Until recently, little was known about factors that trigger CFU-Fs into proliferation and support growth of their immediate progeny. Recently, systems have been developed which allow direct studies of MSF growth control *in vitro* by means of cell separation techniques and defined culture conditions. These studies have revealed the involvement of non-adherent marrow cells, as well as certain growth factors, in the stimulation of MSF growth (Kimura *et al*, 1988, 1989; Friedenstein *et al*, 1992; Gronthos & Simmons, 1995). Many questions still remain, especially when considering that the mechanisms controlling MSF proliferation may differ from one species to another (Kuznetsov & Gehron Robey, 1996).

In the present paper we have studied the control of MSF proliferation *in vitro* by developing a novel assay where contact with serum and with non-adherent marrow cells was limited to the short period required for cell attachment. Subsequent MSF colony formation occurred in conditions free of both serum and non-adherent marrow cells. This approach has enabled us to study directly the effect of various supplements on MSF colony formation. The results indicate that MSF colony formation is a complex process involving at least four growth factors: PDGF, bFGF, TGF-β, and EGF. Mouse and human CFU-Fs differ in their requirements for each of the growth factors for their initial proliferation.

METHODS

Marrow cell suspensions. 6–10-week-old CBA/JCR or FVB/N mice, and 5–10-week-old Hartley guinea-pigs (Charles River Laboratories, Raleigh, N.C.) were killed by CO_2 inhalation in compliance with 'Care and Use of Animals' (small animal protocol 84-92). Femora, tibiae and humera were aseptically removed, and the entire bone marrow content of medullary cavities was flushed with α-modified Minimum Essential Medium (αMEM, Life Technologies, Grand Island, N.Y.). Fragments of normal human bone derived from femoral neck or ileum were obtained from patients of different ages (4·5–60 years) during the course of corrective surgery. All human samples were collected in accordance with the National Institutes of Health (NIH) regulations governing the use of human subjects under protocol 94-D-0188. Pieces of trabecular bone with bone marrow were scraped with a steel blade into the medium and washed until the bone became marrow-free. To prepare single cell suspensions, mouse, guinea-pig and human marrow preparations were pipetted, passed through needles of decreasing diameter (gauges 16 and 20) and subsequently filtered through a 2350 cell strainer (Becton Dickinson, Franklin Lakes, N.J.). Mouse and human marrow cells were plated into 25 cm^2 plastic culture flasks (Becton Dickinson) in 5 ml of medium, at the following initial numbers: for mouse $6–15\times10^5$, for human $1–6\times10^5$ nucleated cells per flask. These cell numbers were calculated from results of preliminary experiments and were utilized in order to (1) permit clonal growth of MSFs, (2) allow the subsequent removal of practically all non-adherent cells as described below, and (3) still give rise to equal numbers of MSF colonies per flask for both species at a sufficient level for statistical analysis.

Preparation of cultures. In order to limit the stimulatory effects of non-adherent marrow cells and serum during MSF colony formation, the preparation of cultures was divided into three steps: adhesion, washing, and cultivation. In the adhesion step, the plated marrow cells were incubated for 3 h at 37°C in serum-containing medium to allow attachment of adherent cells. The serum-containing medium consisted of αMEM, glutamine (2 mM), penicillin (100 U/ml), streptomycin sulphate (100 μg/ml, all Biofluids, Rockville, Md.), and 20% fetal bovine serum (FBS, Becton Dickinson, Franklin Lakes, N.J., or Atlanta Biologicals, Norcross, Ga.) from pre-selected lots. After adhesion, unattached cells were removed by aspiration, and cultures were washed vigorously three times with DMEM (Biofluids, Rockville, Md.). At the cell densities employed in this study, no more than several hundred non-adherent cells per flask were left after the washing step.

After washing, the cultivation step was conducted in either serum-free medium (SFM) or serum-free conditioned medium (SF-CM). SFM consisted of αMEM, glutamine, penicillin, streptomycin sulphate, dexamethasone (10^{-8} M) (Sigma, St Louis, Mo.), L-ascorbic acid phosphate magnesium salt n-hydrate (10^{-4} M) (Wako, Osaka, Japan), and 0·5% ITS$^+$ (Collab. Biomed. Prod., Bedford, Mass.) which contained: insulin 12·5 mg, transferrin 12·5 mg, selenous acid 12·5 μg, bovine serum albumin 2·5 g, linoleic acid 10·7 mg per 20 ml. SF-CM was prepared by incubating SFM with 5×10^6 nucleated guinea-pig bone marrow cells per 1 ml at 37°C for 4 d. It was found that SF-CM generated for 10–96 h, had essentially equal stimulating activity. After incubation, SF-CM was centrifuged at 570 *g* for 20 min and the supernatant was filtered through a 0·22 μm Millipore filter (Millipore Corporation, Bedford, Mass.) to remove particulate matter. SF-CM was either used on the same day or aliquoted and stored at −70°C for up to 9 months.

Supplements to the cultivation media. Supplements were added to the cultivation media just after the washing step. In cultures with SFM without supplements, no MSF colonies were formed (see below). On this negative background, the stimulating effect of feeder cells or growth factors was studied.

Feeder cells ($1·0–1·5\times10^7$ nucleated cells per flask) were guinea-pig bone marrow single-cell suspensions, γ-irradiated with 6000 cGy to prevent cell proliferation. In preliminary experiments it was shown that the stimulating effect of feeder cells was species non-specific: feeder cells of guinea-pig or mouse origin provided a comparable stimulation of MSF colony formation. Guinea-pig bone marrow was used as a source of both feeder cells and SF-CM. It was also found that in mouse cultures maximum MSF colony

numbers were reached at $1{\cdot}0-1{\cdot}5\times10^7$ guinea-pig feeder cells per 25 cm^2 flask. Increasing feeder cell number did not further stimulate colony formation, and feeder cell numbers $>3\times10^7$ suppressed it.

Growth factors were added to SFM separately or in combination. The growth factors used were: human purified TGF-β_1, human recombinant TGF-β_2 (both Genzyme Corp., Cambridge, Mass.), and porcine purified TGF-β_1 (R&D Systems, Minneapolis, Minn.), 0·01–10 ng/ml; human purified PDGF (A-B heterodimer), and porcine purified PDGF (B-B homodimer, both R&D Systems), 1–25 ng/ml; human recombinant bFGF (Collab. Biomed. Prod., Bedford, Mass.), 0·15–150 ng/ml, bovine purified bFGF (R&D Systems), 0·01–10 ng/ml; mouse purified EGF, 1–100 ng/ml; human recombinant IL-6, 20–2000 U/ml; human recombinant IGF-I, 2–200 ng/ml; human recombinant IGF-II (all Collab. Biomed. Prod.), 0·5–50 ng/ml.

In cultures with SF-CM, MSF colonies were formed (see below). On this positive background, the inhibiting effect of antibodies against certain growth factors was studied. The following antibodies were used: mouse monoclonal anti-TGF-β_1,β_2,β_3 to recognize bovine, mouse and human TGF-β_1 and TGF-β_2, and chicken TGF-β_3 (Genzyme Corp.), 1–25 μg/ml; rabbit polyclonal neutralizing anti-human PDGF neutralizing the biological activity of natural human PDGF-AB, natural porcine PDGF-BB, recombinant human PDGF-AB, recombinant human PDGF-BB, and, to a lesser extent, recombinant human PDGF-AA, 1–25 μg/ml; goat polyclonal neutralizing anti-human bFGF (both R&D Systems), 0·4–10 μg/ml; rabbit polyclonal anti-mouse EGF (Collab. Biomed. Prod.), 2–50 μg/ml; normal rabbit IgG (R&D Systems), 20 μg/ml. To control specificity of the inhibiting effect of the antibodies, excess amounts of the corresponding ligands were added to some cultures together with the antibodies. As such, the following growth factors in concentrations exceeding $5\times ED_{50}$ were used: human recombinant TGF-β_2 10 ng/ml, human purified PDGF 25 ng/ml, human recombinant bFGF 15 ng/ml, mouse purified EGF 100 ng/ml.

Culture conditions, fixation, statistical analysis. After cultivation medium, either with or without supplements, was added at day 0, no other medium replacements were carried out. Cultivation was performed at 37°C in a humidified mixture of 5% CO_2 with air. Cultures were fixed on days 11–14 with methanol and stained with an aqueous solution of saturated methyl violet (Sigma, St Louis, Mo.). Some cultures were stained for the presence of α-naphthyl acetate esterase and acid phosphatase activity (Sigma kits 91-A and 387-A, respectively). Colonies containing 30 or more MSFs were counted using a dissecting microscope, and colony forming efficiency (number of MSF colonies per 1×10^5 marrow cells plated) was determined. Analysis of variance was performed and post test comparison was done using the Bonferroni multiple comparison test. Differences were considered statistically significant at $P < 0{\cdot}05$.

Transplantation of MSFs into immunodeficient mouse recipients. To determine whether MSFs grown in serum-free conditions are functionally equivalent to those generated in the presence of serum, their ability to form bone and a haemopoietic microenvironment after transplantation *in vivo* was tested. Mouse and human marrow cells were cultured in SF-CM as described above. After 13 d, the cultures were washed twice with Hanks Balanced Salt Solution and treated with two consecutive portions of 1 × trypsin-EDTA (both Life Technologies). Detached cells were pelleted by centrifugation at 135 *g* for 10 min and loaded into transplantation vehicles according to a technique described in detail elsewhere (Krebsbach *et al*, 1997). Briefly, the transplantation vehicles were composed of 40 mg hydroxyapatite/tricalcium phosphate ceramic (HA/TCP) powder (Zimmer, Warsaw, Ind.). MSFs at $1{\cdot}2-1{\cdot}8\times10^6$ cells per vehicle were attached to the particles by 60–90 min adhesion at 37°C with rotation (25 rpm). Empty vehicles and those bearing human foreskin fibroblasts (of the third passage, kindly provided by Dr Mark DeNichilo) were implanted as controls.

Immunodeficient 12-week-old female beige mice (NIH-bg-nu-xidBR, Harlan Sprague Dawley, Indianapolis, Ind.) were used as subcutaneous implant recipients. Operations were performed in accordance to specifications of an approved small animal protocol (114-93) under anaesthesia achieved by intraperitoneal injection of xylazine (1 mg/ml, Phoenix Pharmaceutical Inc., St Joseph, Mo.) and ketamine hydrochloride (10 mg/ml, Fort Dodge Laboratories Inc., Fort Dodge, Iowa) solution in 0·9% NaCl at 0·01 ml/g of body weight. A mid-longitudinal skin incision was made on the dorsal side of each mouse and subcutaneous pockets were formed by blunt dissection. A single transplant was placed into each pocket with up to four transplants per animal. The incisions were closed with surgical staples. The transplants were recovered 6 weeks after transplantation, fixed and partially decalcified in Bouin's solution (Sigma, St Louis, Mo.), and paraffin embedded. Sections were deparaffinized, hydrated, and stained with haematoxylin and eosin.

RESULTS

MSF colony formation in serum-free conditions

We have previously observed that under optimal culture conditions which include 20% FBS, and in mouse cultures, also in the presence of irradiated marrow feeder cells, most MSF colonies consist of several hundred to several thousand cells after 11–14 d. Some colonies have a multilayer structure and exhibit, in mouse cultures, an accumulation of lipid-laden cells, and, in human cultures, densely organized nodule-like formations (Friedenstein *et al*, 1992; Kuznetsov & Gehron Robey, 1996).

In the current study, marrow cells were first allowed to adhere in serum-containing medium, but subsequent cultivation was carried out in serum-free conditions. In SFM without supplements, no MSF colonies were formed by either mouse or human cells, indicating that any serum factors bound to the cells or plastic are not sufficient to stimulate colony formation. Only scattered macrophages and occasional single fibroblasts could be seen at the end of the cultivation period. In SFM containing guinea-pig feeder cells, MSF colonies were formed; most of which had a monolayer structure and contained 50–200 cells. MSFs featured extensive flattened cytoplasm and a large oval

nucleus with prominent nucleoli; they were negative for both α-naphthyl acetate esterase and acid phosphatase. Macrophage-like cells were abundant in mouse cultures, but much less prominent in human cultures. They could be distinguished from MSF colonies by cell morphology, including much smaller size, round, stellate or bipolar cell shape, small dark nucleus without visible nucleoli, by high activity of α-naphthyl acetate esterase and acid phosphatase, and by a scattered growth pattern. In SFM plus feeder cells, MSF colony forming efficiency (per 1×10^5 marrow cells, mean ± SEM) was 6·1 ± 1·1 for mouse (range 1·5–15·3; six experiments, 14 flasks), and 18·3 ± 4·6 for human cultures (range 6·0–46·5; five experiments, 12 flasks).

In SF-CM (SFM conditioned by guinea-pig marrow cells) without supplements, MSF colonies were also formed. Some colonies contained 50–200 cells, whereas others consisted of only 30–50 cells. Colony forming efficiency was 3·0 ± 0·4 for mouse (range 0·5–6·2; six experiments, 18 flasks), and 19·2 ± 3·2 for human (range 7·0–40·5; five experiments, 12 flasks), which is 49·6 ± 4·9% ($P < 0·001$) and 102·4 ± 12·9% ($P > 0·05$), respectively, of the values in cultures in SFM plus feeder cells. Thus, in serum-free conditions, marrow cells or their products (SF-CM) were necessary for MSF colony formation in both mouse and human cultures. In this study, we were unable to completely avoid serum. If serum was absent at all culture stages, including adhesion and cultivation, no MSF colonies were formed in either mouse or human cultures, no matter what other supplements, including feeder cells or SF-CM, were added.

The stimulating effect of SF-CM was preserved after filtration and after freezing and thawing. If the mean values of MSF colony forming efficiency in cultures with freshly prepared, non-filtered SF-CM were designated as 100%, then in cultures with SF-CM filtered through 0·22 μm Millipore filter, the values were (mean ± SEM) 104·1 ± 15·3% for mouse (eight flasks, $P > 0·05$), and 108·9 ± 4·7% for human cultures (nine flasks, $P > 0·05$). In mouse cultures with frozen and thawed SF-CM the value was 92·7 ± 10·8% (15 flasks, $P > 0·05$). SF-CM could be stored at −70°C for at least 9 months without significant loss of stimulating activity.

The continuous presence of SF-CM was required after day 1 for full stimulating activity. When cells were first cultured in SF-CM, and after 1, 3 or 6 d of cultivation switched to SFM, human MSF colony forming efficiency was greatly decreased. In addition, when cells were first cultured in SFM which was then replaced by SF-CM on days 3 or 6, colony forming efficiency also was profoundly decreased (Fig 1). These results suggest that CFU-F and its immediate progeny need stimulating factor(s) that are present in SF-CM, but absent in SFM, for stimulation of proliferation. In SFM, CFU-Fs do not proliferate and gradually lose the ability to be stimulated to proliferation.

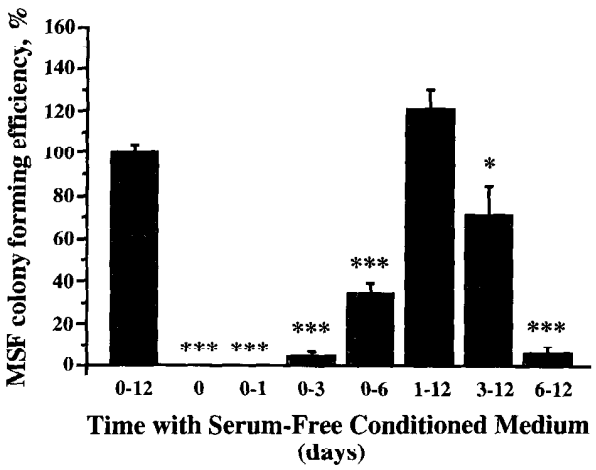

Fig 1. Effect of SF-CM exposure time on MSF colony forming efficiency. 4×10^5 human marrow cells were plated per 25 cm^2 flask. After 3 h of adhesion in serum-containing medium, followed by extensive washing, cultivation medium was added at time 0. Cultivation medium was either SF-CM for the periods of time shown, or SFM for the rest of cultivation (fixation on day 12). Values of MSF colony forming efficiency are shown as percentages from the mean value of the first group where SF-CM was present throughout the cultivation period, which is designated as 100%. Each bar represents mean + SEM of five flasks. Statistically significant differences, from the first group: $^*P < 0·05$; $^{***}P < 0·001$.

Effect of antibodies against growth factors

Although SFM does not support MSF colony formation, the same medium conditioned by guinea-pig marrow cells, SF-CM, becomes supportive. Thus, stimulating activity(s) is released by marrow cells into the medium. In order to determine the required growth factors present in SF-CM, we used neutralizing antibodies against known growth factors and examined their ability to inhibit MSF colony formation. In mouse cultures it was found that all four antibodies tested decreased MSF colony formation in a dose-dependent fashion (Fig 2). Rabbit anti-human PDGF almost completely abolished MSF colony formation at 25 μg/ml, and profoundly decreased it at 1 and 5 μg/ml. MSF colony formation was significantly inhibited by goat anti-human bFGF at 2 and 10 μg/ml, and by rabbit anti-mouse EGF at 10 and 50 μg/ml. The effect of mouse monoclonal anti-TGF-$\beta_1\beta_2\beta_3$ was not as pronounced with only the highest concentration, 25 μg/ml, significantly decreasing MSF colony numbers. Normal rabbit IgG had no effect. Excess of the corresponding ligands reversed, either completely or partially, the inhibiting effect of each of the antibodies (Fig 2), thus demonstrating the specificity of their action on MSF colony formation.

In human cultures, the same concentrations of the antibodies were tested. All four antibodies significantly decreased MSF colony formation, but the degree of suppression differed from mouse (Fig 3). Anti-PDGF had an effect similar to that in mouse cultures, a profound dose-dependent suppression of colony formation that was almost complete at 5 and 25 μg/ml. The inhibiting effect of anti-TGF-β was much more prominent in human than in mouse cultures. On the contrary, the effect of anti-EGF and, especially, of anti-bFGF was much less pronounced than in mouse cultures. The excess of the corresponding ligands substantially restored MSF colony formation (Fig 3).

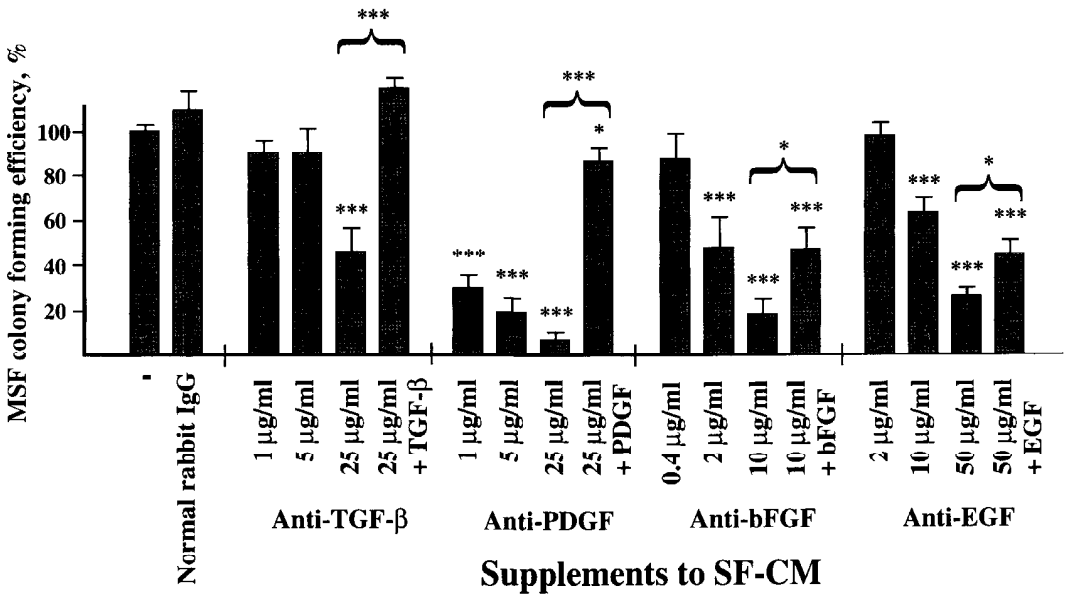

Fig 2. Effect of antibodies against growth factors on mouse MSF colony forming efficiency. $10-12\times10^5$ mouse marrow cells were plated per 25 cm^2 flask. After 3 h of adhesion in serum-containing medium and extensive washing, SF-CM was added, with or without antibodies at the concentrations shown, corresponding growth factors, or normal IgG (20 μg/ml). The growth factors were added at the following concentrations: human TGF-β_2 10 ng/ml, human PDGF 25 ng/ml, human bFGF 15 ng/ml, mouse EGF 100 ng/ml. The values of MSF colony forming efficiency are shown as percentages from the mean value of cultures in SF-CM without supplements, which is designated as 100%. Each bar represents mean + SEM of four to six cultures. Statistically significant differences, from cultures in SF-CM without supplements: $^*P < 0.05$; $^{***}P < 0.001$. Statistical differences between the two groups with the highest concentration of a particular antibody, with or without excess of the corresponding ligand, are designated over the brackets.

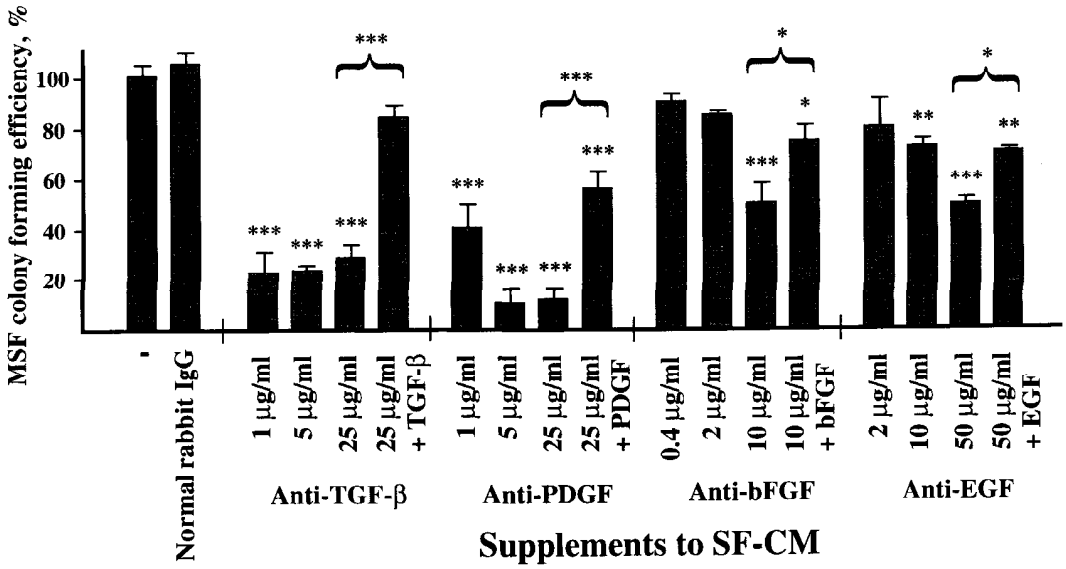

Fig 3. Effect of antibodies against growth factors on human MSF colony forming efficiency. $4-5\times10^5$ human marrow cells were plated per 25 cm^2 flask. After 3 h of adhesion in serum-containing medium and extensive washing, SF-CM was added, with or without antibodies at the concentrations shown, corresponding growth factors, or normal IgG (20 μg/ml). The growth factors were added at the same concentrations as shown in Fig 2. The values of MSF colony forming efficiency are shown as percentages from the mean value of cultures in SF-CM without supplements, which is designated as 100%. Each bar represents mean + SEM of four cultures. Statistically significant differences, from cultures in SF-CM without supplements: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Statistical differences between the two groups with the highest concentration of a particular antibody, with or without excess of the corresponding ligand, are designated over the brackets.

Effect of growth factors

Based on the results of neutralizing antibody experiments, we tried to reconstitute the stimulating activity(s) released by marrow cells. For this purpose, several growth factors were added into SFM, separately or in combination. In mouse cultures, only bFGF alone was able to partially substitute for the stimulating activity of SF-CM. Both human recombinant bFGF and bovine purified bFGF were active at low or intermediate concentrations but not at higher ones (Fig 4b). None of the other growth factors, including human and

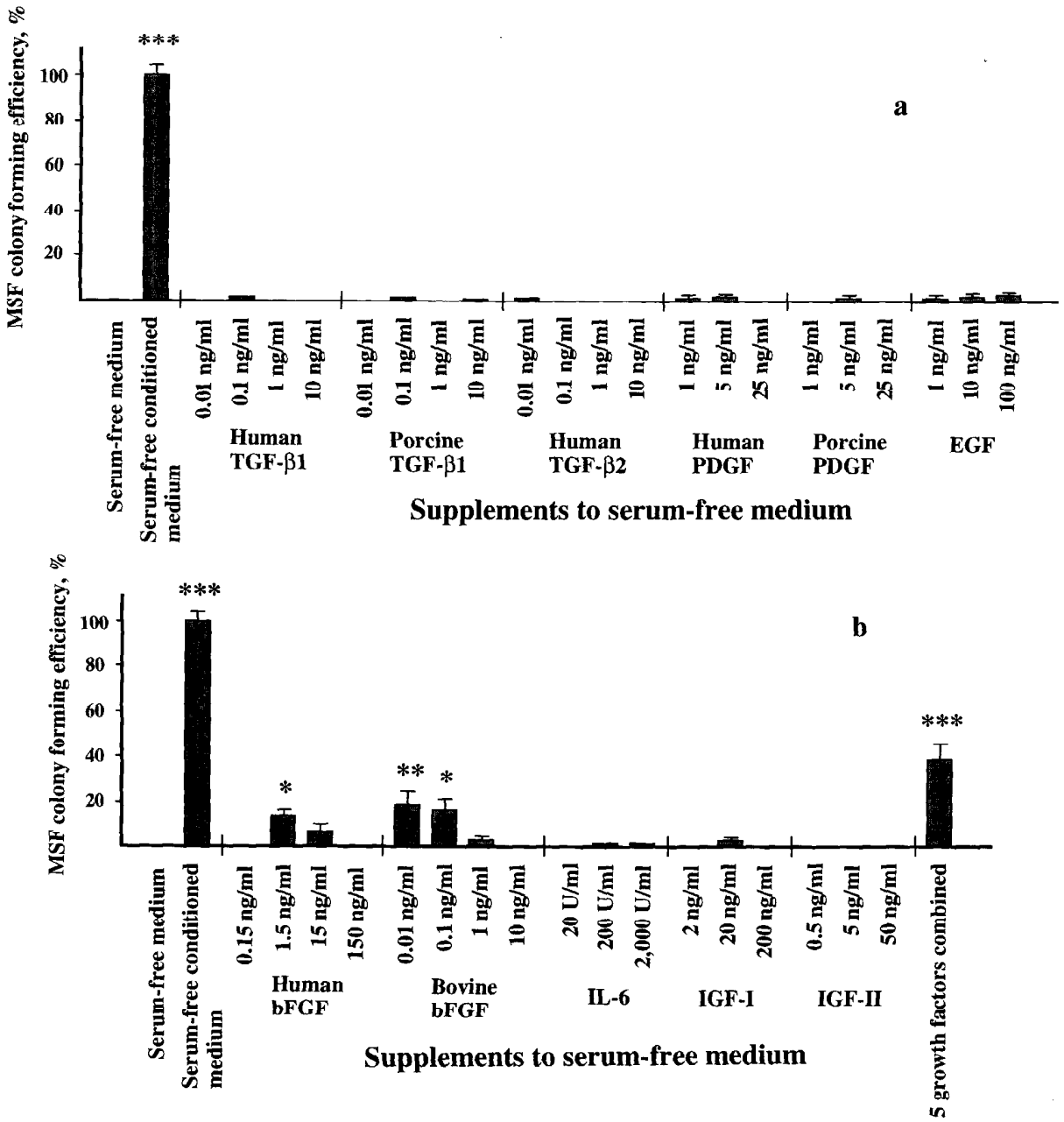

Fig 4. Effect of growth factors on mouse MSF colony forming efficiency. $10–15 \times 10^5$ mouse marrow cells were plated per 25 cm^2 flask. After 3 h of adhesion in serum-containing medium and extensive washing, cultivation medium was added which was either SFM, with or without growth factors at the concentrations shown, or SF CM. To the last group, the combination of five growth factors was added. These growth factors were: porcine TGF-β1 10 ng/ml, porcine PDGF 5 ng/ml, EGF 100 ng/ml, human bFGF 1·5 ng/ml, IGF-I 20 ng/ml. Values of MSF colony forming efficiency are shown as percentages from the mean value of cultures in SF-CM, which is designated as 100%. Each bar represents mean + SEM of four flasks (most growth factors) or of six to eight flasks (groups with bFGFs and with five growth factors). Statistically significant differences, from cultures in SFM: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

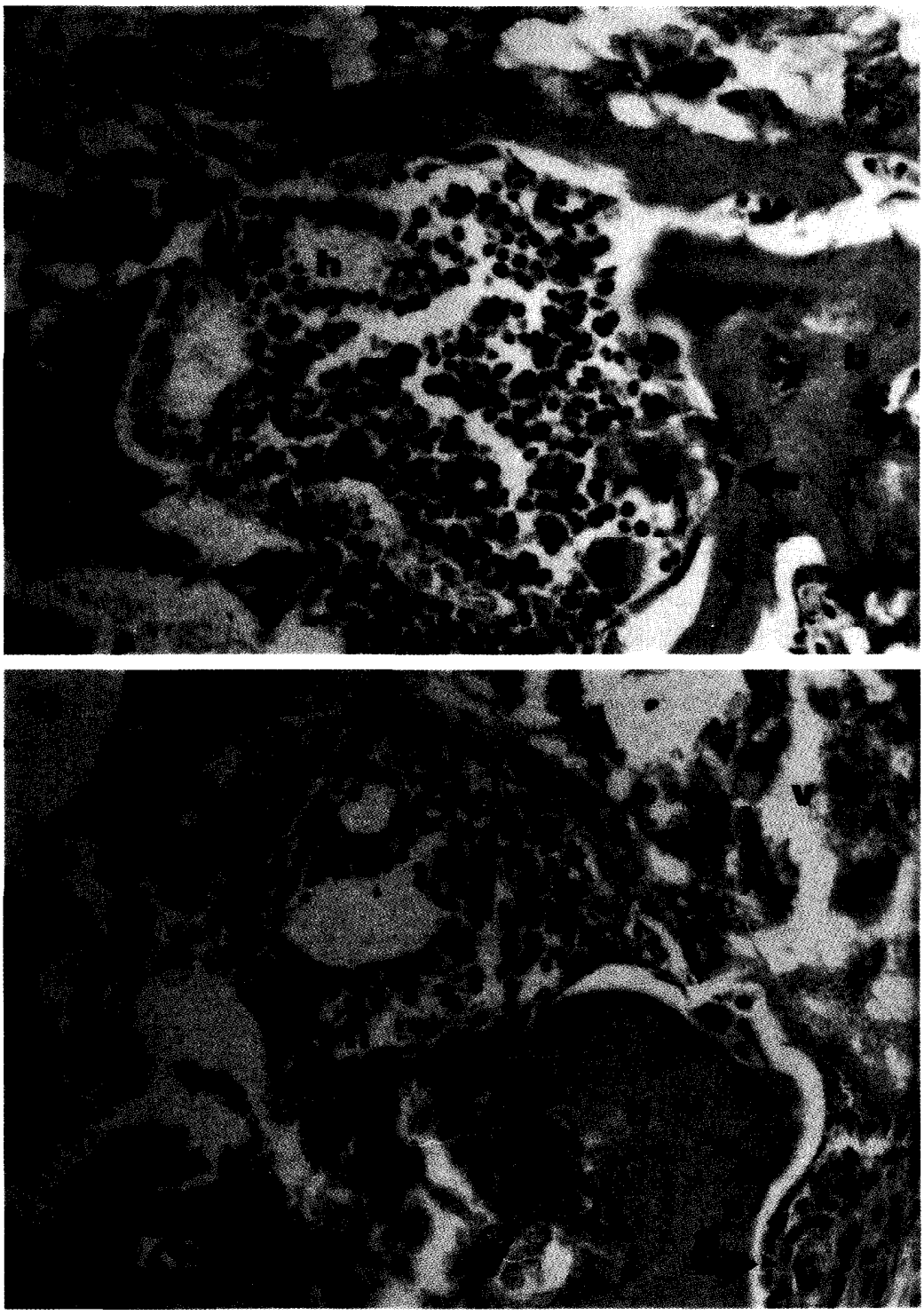

Fig 5. *In vivo* implants of MSFs grown in serum-free conditions. Mouse (A) or human (B) marrow cells were grown in serum free conditioned medium, as described in Methods. After 13 d, MSFs were harvested with trypsin, loaded into hydroxiapatite/tricalcium phosphate ceramic powder, and implanted subcutaneously into immunodeficient mice. Six weeks later the implants were found to contain bone trabeculae (b) surrounding vehicle particles (v); haemopoietic tissue (h) and fibrous tissue (f) are adjacent to the new bone. Note the presence of megakaryocyte-like cells. Large arrows designate osteoblasts; small arrows designate osteocytes. Haematoxylin and eosin, ×180.

porcine TGF-β_1, human TGF-β_2, human and porcine PDGF, mouse EGF, and human IL-6, IGF I and IGF II, showed any significant stimulation of MSF colony formation when tested alone (Fig 4a, b). A combination of five growth factors, including porcine TGF-β_1 at 10 ng/ml, porcine PDGF at 5 ng/ml, EGF at 100 ng/ml, human bFGF at 1·5 ng/ml, and IGF-I at 20 ng/ml, produced a significant stimulation of MSF colony formation (Fig 4b).

In human cultures, the same concentrations of the growth factors were tested as in the mouse experiments. None of the growth factors tested produced any significant stimulation of MSF colony formation (six flasks per group, not shown). Different combinations of five growth factors, including human TGF-β_1 at 0·1–10 ng/ml, human PDGF at 1–25 ng/ml, EGF at 1–100 ng/ml, human bFGF at 1·5–150 ng/ml, and IGF-I at 2–200 ng/ml, did not stimulate MSF colony formation (not shown).

Ability of MSFs grown in SF-CM to form bone and haemopoietic microenvironment

Six weeks after transplantation, transplants of both mouse and human MSFs grown in SF-CM exhibited extensive bone formation (Fig 5). New bone was deposited against the vehicle particles and showed embedded osteocytes and an osteoblastic layer on lumenal surfaces. Between the bony trabeculae, fibrous cords and fields of haemopoietic tissue closely associated with the new bone could be observed. Haemopoietic cells predominantly consisted of mature granulocytes of mouse origin, judging by their ring-shaped nuclei, although some non-mature cells and megakaryocytes were also present. Three transplants each of mouse and human MSFs were performed, and in all of them new bone and haemopoietic tissue were developed. In transplants of empty vehicles or of those bearing human foreskin fibroblasts, only fibrous tissue could be found with no signs of bone formation or haemopoiesis (not shown).

DISCUSSION

In the current study we have developed a system whereby MSF colony formation occurred in the absence of both serum and non-adherent marrow cells. Serum was present for 3 h during cell adhesion, and was absent thereafter. The use of serum-free medium enabled us to study the effect of growth factors and antibodies against them. The vast majority (>99%) of non-adherent marrow cells was removed from the cultures after the initial adhesion, prior to subsequent cultivation. Non-adherent marrow cells have been shown to directly stimulate MSF proliferation (Castro-Malaspina *et al*, 1980, 1981; Hirata *et al*, 1985; Friedenstein *et al*, 1992; Kuznetsov & Gehron Robey, 1996) and to mediate effects of exogenous substances (Rickard *et al*, 1995). Therefore the removal of non-adherent cells was necessary to ensure that the disclosed effects of SF-CM or growth factors on MSF proliferation were direct rather than mediated through other cell types.

In serum-free medium without supplements, no MSF colonies were formed in either mouse or human cultures. MSF colony formation was restored when guinea-pig marrow cells (irradiated to prevent their proliferation), or their products in the form of serum-free conditioned medium, were added. The stimulating activity produced by guinea-pig marrow cells was species non-specific; did not require direct cell contact; was not altered by filtration through 0·22 μm Millipore filter; was stable at −70°C; and had to be present in the culture continuously after day 1. In mouse and human cultures in SF-CM, antibodies against PDGF, TGF-β, bFGF and EGF significantly decreased MSF colony formation. The specificity of the inhibiting effect was demonstrated by the fact that excess of the corresponding growth factors abolished the inhibition. These data imply that all four growth factors actually participate in, and are necessary for, initial proliferation of both CFU-F and its immediate descendants. In mouse cultures, the most prominent inhibition was rendered by anti-PDGF, an intermediate level by anti-bFGF and anti-EGF, and anti-TGF-β was the least effective. In human cultures, the suppressing effect of anti-PDGF was also very profound. However, the effect of anti-TGF-β was much more pronounced, and the effect of anti-bFGF and anti-EGF was less pronounced than in mouse cultures. The various effects of the antibodies on mouse and human MSF colony formation implied that mouse and human CFU-Fs needed different concentrations of individual growth factors to begin proliferation. This may be due to differences in the levels of the corresponding receptors on mouse and human CFU-Fs.

In mouse cultures in SFM, bFGF alone stimulated MSF colony formation. When bFGF was added together with a mixture of other growth factors, MSF colony formation was further stimulated. Other growth factors, including TGF-β_1, TGF-β_2, PDGF, EGF, IL-6, IGF-I and IGF-II, had no effect when tested alone. Interestingly, mouse MSF colony formation was stimulated by low or intermediate concentrations of both human recombinant bFGF and bovine purified bFGF, whereas higher concentrations had no effect. It is possible that high bFGF concentrations caused the prevention of receptor dimerization by saturating them as 1 : 1 complexes (Wells, 1994). In human cultures in SFM, none of the growth factors tested, either separately or in combination, supported MSF colony formation. These data suggest, once again, that mouse and human CFU-Fs have different growth factor requirements. In serum-free conditions, human CFU-Fs are more demanding than mouse CFU-Fs. They are not stimulated by bFGF and apparently need some other component(s) secreted into SF-CM by marrow cells. Conversely, in serum-containing conditions, human CFU-Fs have been shown to form colonies without any additional stimuli, whereas mouse CFU-Fs are more demanding and require the presence of non-adherent marrow cells for proliferation (Kuznetsov & Gehron Robey, 1996).

The findings reported here are somewhat different from the results of Gronthos & Simmons (1995) who have shown that in human marrow cell primary cultures only PDGF and EGF stimulate MSF colony formation, whereas bFGF and TGF-β have no effect. Our results also differ from reports that in human primary cultures (in the absence of non-adherent marrow cells), PDGF and EGF, but not TGF-β, stimulate MSF colony formation (Kimura *et al*, 1988). In the first of the

studies, MSF colony assay was performed in a serum-free medium which differed from our medium in several aspects, including a concentration of BSA (2%) that is 32 times higher, and a concentration of human transferrin (100 μg/ml) that is 64 times higher. None of the two products were recombinant, and therefore both may contain traces of multiple serum activities. In the second study, the assay was performed in medium containing 5% human serum which was derived from platelet-poor plasma and included an undefined combination of serum components. The results reported in these two studies suggest that, under their particular culture conditions, only PDGF and EGF were below a critical concentration although presumably other necessary growth factors were present as contaminants of the BSA and transferrin preparations. The concentration of components of our serum-free medium was considerably lower. Consequently, some other activities discharged by marrow cells were needed in addition to PDGF and EGF to trigger human CFU-Fs into proliferation. Earlier data support our conclusion of the role played by TGF-β and bFGF in human MSF growth control. PDGF and bFGF were shown to stimulate proliferation of passaged human MSFs, whereas EGF had no effect, and TGF-β either stimulated or inhibited proliferation depending on its concentration and cell density (Kimura *et al*, 1989; Oliver *et al*, 1990). These data may also imply that during continuous proliferation *in vitro* (i.e. after stimulation of initial proliferation) human MSFs alter their growth factor requirements.

Limited data are available concerning growth factor requirements for mouse MSF colony formation. Previously we reported that, in the absence of non-adherent marrow cells, no MSF colonies were formed in mouse cultures with 20% FBS. In these conditions, neither PDGF nor EGF could stimulate colony formation (Friedenstein *et al*, 1992) as it is described here for serum-free medium. In the presence of non-adherent marrow cells, mouse MSF colonies are formed without exogenous growth factors, but colony numbers are increased by IL-3 and, at different serum concentrations, by either PDGF or bFGF (Wang *et al*, 1990). These findings demonstrate that mouse MSF growth requirements are complex and may be better understood when studied without serum components and non-adherent marrow cells. The complexity and species-dependent character of MSF growth regulation is further illustrated by the observations that proliferation of rat MSFs is stimulated by bFGF and inhibited by TGF-β (Pitaru *et al*, 1993; Locklin *et al*, 1995).

The question arises whether MSFs grown in serum-free conditions are functionally equivalent to MSFs generated in the presence of serum. To address this issue, we transplanted MSFs grown in SF-CM into immunodeficient mice *in vivo*. In the implants recovered 6 weeks after transplantation, extensive formation of new bone accompanied by a haemopoietic microenvironment was observed. We have shown earlier that mouse and human MSFs grown in conventional serum-containing conditions form bone in virtually 100% of the implants containing HA/TCP ceramics (Krebsbach *et al*, 1997). It has been also demonstrated by our group and others that in heterotopic transplants of marrow cells, including MSFs, bone and stromal tissue are of donor origin, whereas haemopoietic cells originate from recipients (Friedenstein *et al*, 1978; Hotta *et al*, 1983; Haynesworth *et al*, 1992; Krebsbach *et al*, 1997). The findings presented here demonstrate for the first time that MSFs grown in serum-free medium are similar to MSFs grown in standard serum-containing conditions in their capacity to differentiate towards bone formation and support of haemopoiesis. These data imply that no selective elimination of a particular CFU-F subpopulation occurs in serum-free conditions, and therefore the results reported here are likely to be representative of the total CFU-F population.

In conclusion, it has been shown that at least four growth factors, PDGF, bFGF, TGF-β and EGF, are necessary for MSF colony formation *in vitro*. The requirements for the individual growth factors are species-dependent and differ between mouse and human CFU-Fs. Yet the question remains whether CFU-F proliferation *in vivo* is regulated by the same molecules as those described here and elsewhere for *in vitro* models, and is a topic for further study.

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